Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Canine Distemper Virus in Vero Cell Culture

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) is an *in vitro* test method for assaying the viral content of modified-live canine distemper virus (CDV) vaccines. This test is applicable to CDV vaccines when the master seed virus (MSV) has been adapted to African green monkey kidney (Vero) cells and the MSV produces cytopathic effect (CPE).

1.2 Keywords

Canine distemper virus, CDV, $TCID_{50}$, *in vitro* potency test, titration, Vero cells, CPE, 9 CFR 113.306

2. Materials

2.1 Equipment/instrumentation

2.1.1 36° ± 2°C, high humidity, 5% ± 1% CO_2 incubator[1] meeting the requirements of the current version of GDOCSOP0004

2.1.2 36° ± 2°C water bath[2] meeting the requirements of the current version of GDOCSOP0002

2.1.3 Inverted light microscope[3]

2.1.4 Vortex mixer[4]

2.1.5 Self-refilling repetitive syringe, 2 ml[5]

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[1] Model 3336, Forma Scientific Co., P.O. Box 649, Marietta, OH 45750 or equivalent
[2] Cat. No. 66648, Precision Scientific Co., 3737 West Cortland St., Chicago, IL 60647 or equivalent
[3] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent
[4] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent
[5] Wheaton, Cat. No. 13-689-50C, Fisher Scientific Corp., 319 W. Ontario, Chicago, IL 60610 or equivalent

2.1.6 Pipettor[6] with tips[7] and/or motorized microliter pipette[8] and tips[9]

2.1.7 Micropipettor[10], 300 µl x 12 channel

2.1.8 Pipette-aid[11]

2.2 Reagents/supplies

2.2.1 CDV Reference, Onderstepoort strain[12]

2.2.2 Monospecific antisera,[12] free of CDV antibody, that neutralize the non-CDV fractions present in multifraction vaccines, e.g., canine parainfluenza virus (CPI) and canine adenovirus (CAV)

2.2.3 Vero cells[13] free of extraneous agents as tested by the Code of Federal Regulations, Title 9, (9 CFR)

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM with Earle's salts[14]

2.2.4.2 2.2 g sodium bicarbonate[15]

2.2.4.3 Dissolve with 900 ml deionized water (DW).

2.2.4.4 Add 5.0 g lactalbumin hydrolysate or edamin[16] to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to the solution in **Section 2.2.4.3** with constant mixing.

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[6] Cat. No. P-200, Rainin Instrument Co., P.O. Box 4026, Mack Rd., Woburn, MA 01801-4628 or equivalent
[7] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent
[8] Cat. No. E2-1000, Rainin Instrument Co. or equivalent
[9] Cat. No. RT-200, Analytic Lab Accessories or equivalent
[10] Finnpipettes, Cat. No. NV204662D, A. Daigger Co., Inc., 199 Carpenter Ave., Wheeling, IL 60090 or equivalent
[11] Cat. No. 183, Drummond Scientific Co., 500 Pkwy., Broomall, PA 19008 or equivalent
[12] Reference quantities are available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent
[13] ATCC CCL 81, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852
[14] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[15] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[16] Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

2.2.4.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[17]

2.2.4.6 Sterilize through a 0.22-µm filter.[18]

2.2.4.7 Aseptically add:

1. 25 units/ml penicillin[19]

2. 50 µg/ml gentamicin sulfate[20]

3. 100 µg/ml streptomycin[21]

2.2.4.8 Store at 4° ± 2°C.

2.2.5 Growth Medium

2.2.5.1 940 ml MEM

2.2.5.2 Aseptically add:

1. 50 ml gamma irradiated fetal bovine serum (FBS)

2. 10 ml L-glutamine[22]

2.2.5.3 Store at 4° ± 2°C.

2.2.6 Dulbecco's phosphate buffered saline (DPBS)

2.2.6.1 8.0 g sodium chloride (NaCl)[23]

2.2.6.2 0.2 g potassium chloride (KCl)[24]

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[17] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
[18] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[19] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent
[20] Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[21] Cat. No. S-9137, Sigma Chemical Co. or equivalent
[22] 200 mM (100X) liquid, Cat. No. G-7513, Sigma Chemical Co. or equivalent
[23] Cat. No. 3624-01, J.T. Baker, Inc. or equivalent
[24] Cat. No. P217-500, Fisher Scientific Co., 2000 Park Ln., Pittsburgh, PA 15275 or equivalent

2.2.6.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)[25]

2.2.6.4 0.1 g magnesium chloride, hexahydrate ($MgCl_2 \cdot 6H_2O$)[26]

2.2.6.5 Dissolve reagents with 900 ml DW.

2.2.6.6 Add 1.03 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[27] to 10 ml DW, heat to $60^0 \pm 2^0$C until dissolved, then add to **Section 2.2.6.5** with constant mixing.

2.2.6.7 Dissolve 0.1 g calcium chloride, anhydrous ($CaCl_2$)[28] with 10 ml DW and add slowly to **Section 2.2.6.6** to avoid precipitation.

2.2.6.8 Q.S. to 1000 ml with DW, adjust pH to 7.0-7.3 with 2N HCl.

2.2.6.9 Sterilize through a 0.22-µm filter.

2.2.6.10 Store at 4° ± 2°C.

2.2.7 Polystyrene tubes, 12 x 75 mm[29]

2.2.8 Pipettes, 10 ml[30]

2.2.9 Reagent reservoir[31]

2.2.10 Syringe (tuberculin slip tip), 1 ml[32]

2.2.11 Needles, 18 ga x 1 1/2 in[33]

2.2.12 Cell culture plates, 96 well[34]

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[25] Cat. No. 3246-01, J.T. Baker, Inc. or equivalent
[26] Cat. No. M33-500, Fisher Scientific Co. or equivalent
[27] Cat. No. 3828-01, J.T. Baker, Inc. or equivalent
[28] Cat. No. 4225-05, J.T. Baker, Inc. or equivalent
[29] Falcon 2058, Becton Dickinson Labware, 2 Oak Park, Bedford, MA 01730 or equivalent
[30] Falcon 7530, Becton Dickinson Labware or equivalent
[31] Cat. No. 4870, Costar Corp. or equivalent
[32] Cat. No. 309602, Becton Dickinson & Co., 1 Becton Dr., Franklin Lakes, NJ 07417-1884 or equivalent
[33] Cat. No. 305196, Becton Dickinson & Co. or equivalent
[34] Cat. No. 3596, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent

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3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have experience in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses and the quantitation of virus infectivity by CPE.

3.2 Preparation of equipment/instrumentation

On the day of inoculation, set a water bath at 36° ± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of Vero cell culture plates (Vero Plates)

3.3.1.1 Cells are prepared from healthy, confluent Vero cell cultures. On the day of test initiation, using a 12-channel micropipettor and reagent reservoir, seed 100 µl/well in all wells of the 96-well cell culture plates with Vero cells suspended in Growth Medium at a density of approximately $10^{4.7}$ to $10^{5.0}$ cells/ml. Prepare 1 Vero Plate for the controls and the first Test Serial. Each additional Vero Plate allows testing of 2 additional Test Serials.

3.3.1.2 Use the Vero Plates within 4 hr.

3.3.2 Preparation of CDV Reference Control

3.3.2.1 On the day of test initiation, rapidly thaw a vial of CDV Reference in the water bath.

3.3.2.2 Using a 2-ml self-refilling repetitive syringe, dispense 1.8 ml of MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB-L Reference and Reagent sheet; appropriately label (e.g., 5 tubes, labeled 10^{-1} through 10^{-5}, respectively).

3.3.2.3 With a 200-µl pipettor, transfer 200 µl of the CDV Reference to the first tube, labeled 10^{-1}; mix by vortexing.

3.3.2.4 Using a new pipette tip, transfer 200 µl from the 10^{-1} labeled tube (**Section 3.3.2.3**) to the 10^{-2} tube; mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200 µl of the previous dilution to the next dilution tube, until the tenfold dilution series is completed.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). On the day of test initiation, rehydrate a vial of the Test Serial by transferring 1.0 ml for a 1-ml-dose Test Serial, 0.5 ml for a 1/2-ml-dose Test Serial, etc., of the provided diluent into the vial containing the lyophilized Test Serial. Use a sterile 1.0-ml syringe and an 18 ga x 1 1/2-in needle; mix by vortexing. Incubate for 15 ± 5 min at room temperature (RT) (23° ± 2°C).

3.4.2 For multifraction CDV Test Serials, neutralize the non-CDV fractions with antiserum specific to each virus fraction. It is not necessary to neutralize canine parvovirus (CPV) since CPV is not expected to replicate in Vero cells.

3.4.2.1 Prepare 1:2 dilutions of each neutralizing non-CDV antiserum by mixing equal volumes of antiserum and DPBS.

3.4.2.2 Dispense 200 µl of each of the required neutralizing antisera into a 12 x 75-mm polystyrene tube labeled 10^{-1} and q.s. with MEM to 1.8 ml. For example, to neutralize the CPI and CAV components in a 4-fraction CDV/CPV/CPI/CAV vaccine, dispense 200 µl of each of the diluted

CPI and CAV antisera into the tube labeled 10^{-1}; add 1.4 ml of MEM to obtain a final volume of 1.8 ml.

3.4.2.3 Pipette 200 µl of the reconstituted Test Serial to the labeled tube to yield a 10^{-1} dilution; mix by vortexing.

3.4.2.4 Incubate at RT for 30 ± 5 min.

3.4.3 For Test Serials not containing CPI or CAV, the 10^{-1} dilution is prepared by adding 200 µl of the Test Serial to 1.8 ml of MEM in a 12 x 75-mm polystyrene tube, labeled 10^{-1}; mix by vortexing.

3.4.4 Serial tenfold dilutions

3.4.4.1 Dispense 1.8 ml of MEM into each of 4, 12 x 75-mm polystyrene tubes, labeled 10^{-2} through 10^{-5}.

3.4.4.2 Using a new pipette tip, transfer 200 µl from the tube labeled 10^{-1} to the 10^{-2} tube; mix by vortexing.

3.4.4.3 Repeat **Section 3.4.5.2** by transferring 200 µl from the previous dilution to the next dilution tube, until the tenfold dilution series is completed.

4. Performance of the test

4.1 Label the Vero Plates and inoculate each of 5 wells/dilution with 100 µl of dilutions 10^{-5} through 10^{-2} of the Test Serial. In a similar manner, inoculate 5 wells/dilution of the CDV Reference Control (with dilutions 10^{-5} through 10^{-2} from **Section 3.3.2**). Change tips between each unique sample (i.e. each Test Serial and the CDV Reference Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g., 10^{-5} through 10^{-2}).

4.2 Five uninoculated wells serve as negative cell controls.

4.3 Incubate Vero Plates in a 36 ° ± 2°C CO_2 incubator for 168 ± 24 hr.

4.4 After incubation, read the Vero Plate at 100X magnification on an inverted light microscope and examine cells for CDV CPE.

4.4.1 Wells displaying 1 or more CPE foci, characterized by cell fusion and lysis, are considered to be positive for CDV.

4.4.2 Results are recorded as the number of CPE positive wells versus total number of wells examined for each dilution of the Test Serial and the CDV Reference Control.

4.5 Calculate the CDV endpoints of the Test Serial and the CDV Reference Control using the Spearman-Kärber method as refined by Finney. The titers are expressed as $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$).

Example:

10^{-2} dilution of Test Serial = 5/5 wells CPE positive
10^{-3} dilution of Test Serial = 3/5 wells CPE positive
10^{-4} dilution of Test Serial = 1/5 wells CPE positive
10^{-5} dilution of Test Serial = 0/5 wells CPE positive

Spearman-Kärber calculation of total CPE positive wells (9), using 5 wells per dilution = 1.3 log

$\log_{10}$ of reciprocal dilution (10^{-2}) = 2.0 log

$\log_{10}$ of reciprocal of dose factor:

$$\frac{\text{0.1 ml inoculum}}{\text{1 ml dose}} = \frac{1}{10} = 1.0 \log$$

Total = 4.3 log

Titer of the Test Serial is $10^{4.3}$ $TCID_{50}$ per 1-ml dose.

5. Interpretation of the test results

5.1 Valid Assay

5.1.1 The calculated titer of the CDV Reference Control must fall within ± 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The lowest inoculated dilution must exhibit a 100% positive CPE (5/5), and the highest (most dilute) must exhibit no positive CPE (0/5).

5.1.3 The uninoculated cell controls must not exhibit any CPE or cloudy media that would indicate contamination.

5.1.4 If the validity requirements are not met, then the assay is considered a NO TEST and can be retested without prejudice.

5.1.5 If the validity requirements are met and the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service filed Outline of Production for the product under test, the Test Serial is considered SATISFACTORY.

5.1.6 If the validity requirements are met but the titer of the Test Serial is lower than the required minimum titer contained in the Animal and Plant Health Inspection Service filed Outline of Production for the product under test, the Test Serial is retested according to 9 CFR, Part 113.8.

6. Report of test results

Results are reported as $TCID_{50}$ per dose of Test Serial.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.306, U.S. Government Printing Office, Washington, D.C., 1999.

7.2 Cottral GE, (Ed.) *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca and London, 1978, pg 731.

7.3 Finney, DJ *Statistical methods in biological assay*. Griffin, London. 3rd edition, 1978, pg 508.

8. Summary of revisions

This document is new and reflects procedures adopted in the Mammalian Virology Section, CVB-L, to incorporate different methods for testing CDV vaccines.